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Vaccine 18 (2000) 460–467

Vaccine

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Humoral and cellular immune responses to HIV-1 Nef in mice DNA-immunised with non-replicating or self-replicating expression vectors

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Received 28 September 1998; received in revised form 3 May 1999; accepted 14 May 1999

Abstract

Objective: HIV accessory protein Nef is expressed early in the infectious cycle of the virus and has been shown to be an effective immunogen in humoral and cellular immune responses. We have used two different self-replicating pBN vectors and one non-replicating pCGal2 derived (pCG) vector expressing HIV-1 Nef in DNA immunisation of mice in order to determine their efficiency in raising humoral and cellular immune responses. *Design and methods*: The expression of Nef by the three plasmids was tested by transfections into COS-1 cells. Balb/c mice were immunised with the pBN-NEF and pCGE2-NEF constructs using gold particle bombardment. Immunoblotting and immunocytochemistry were used to detect in vitro expression of Nef. ^{51}Cr release assay, ELISA and immunoblotting were used to detect cellular and humoral immune responses in immunised mice. *Results*: Efficient in vitro expression of Nef was detected in pBN and pCGE2-NEF transfected cells, in pBN-NEF transfected cells the expression lasting up to three weeks. Anti-Nef antibodies in sera of 13 of 16 pBN-NEF immunised mice were detected within four weeks after the last immunisation, whereas only 2 of 12 pCGE2-NEF immunised mice had very weak anti-Nef antibodies. Twelve of the pBN-NEF immunised mice (75%) and 6 the pCGE2-NEF immunised mice (50%) showed Nef-specific cytotoxic T lymphocyte (CTL) responses within four weeks. *Conclusions*: We conclude that the three eukaryotic expression vectors tested are capable of inducing a cell mediated immune response towards HIV-1 Nef and should be considered as part of a genetic HIV vaccine.

Keywords: HIV-1 Nef; DNA immunisation; Antibodies; Cytotoxic T-lymphocytes

1. Introduction

One of the main obstacles in the development of an effective HIV vaccine is thought to be the infidelity of the viral reverse transcriptase leading to a rapid evolvement of immune escape mutants, especially when the protective mechanism is based on neutralising antibodies that recognise HIV envelope glycoprotein [1–3]. Cell mediated immunity, if targeted against cells expressing conserved HIV proteins produced in the early phase of viral cycle, such as the regulatory proteins Nef, Rev and Tat, could prevent the release of infectious viral particles and thus partly circumvent the problem caused by mutational variation. Several clinical observations suggest that an effective cytotoxic T

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0264-410X/99/$ - see front matter
PII: S0264-410X(99)00245-5

from the vector, in co-operation with the ORI, facilitate the amplification of the vector sequences in the cell and therefore increase considerably the gene dosage for the expression of the antigen (E. Ustav, A. Männik and M. Ustav, unpublished).

We report here the results obtained when these HIV-1 Nef expressing vectors were used to immunise mice by gene gun inoculation.

2. Materials and methods

2.1. Construction of the expression vectors

Plasmid constructs on which the cloning of HIV genes was based were cloned at Tartu University. pNp177, pUEp1 and pUE83 are cloned from pNp175 (not published, a gift from N. Peunova, Cold Spring Harbor Laboratory, USA). The pNp177 differs from pNp 175 only by an additional *Hin*dIII site. In pUE83 the BPV-1 URR was added to pNp177. For the pUEp1 plasmid an XbaI site at upstream RSV LTR was eliminated and the Lac Z gene was replaced with MCS of pUC19. The pBN series of vectors are derived from the pBabeNeo [22].

2.2. pBN-CMV-NEF

The Nef cDNA fragment of HIV-1(strain BRU) was digested from a plasmid pcNEF (a generous gift from Dr. Brian Cullen, Howard Hughes Medical Institute, Duke University Medical Center, USA) as a SpeI, *Hin*dIII digest (New England Biolabs), and the *Hin*dIII site was filled in to make a blunt end. This fragment was cloned into the shuttle vector pNp177 between XbaI and XhoI sites. A *Hin*dIII cassette of the shuttle vector was then cloned into the *Hin*dIII site of the expression vector pBN. The final construct was called pBN-CMV-NEF, in which the nef gene is under the control of a CMV promoter (Fig. 1).

2.3. pBN-RSV-NEF

The Nef cDNA was amplified by PCR from the plasmid pcNEF using a 5′-primer with an XbaI linker and a 3′-primer with an XhoI linker. The resultant cDNA fragment was cloned into pUE83 shuttle vector and a *Hin*dIII cassette containing the RSV promoter and the nef gene was further cloned into the *Hin*dIII site of the expression vector pBN. The final construct was named pBN-RSV-NEF (Fig. 1).

2.4. pCGE2-NEF

The cDNA encoding the HIV-1 protein Nef was amplified by PCR from the plasmid pcNEF. In PCR, oligonucleotides containing an XbaI linker (5′-primer) and a BamHI linker (3′-primer) together with the dNTP mixture (Boehringer-Mannheim) and Dynazyme TaqDNA polymerase (Finnzymes) were used. The resultant Nef cDNA fragment was then cloned into restriction enzyme sites (XbaI and BamHI, New England Biolabs) of the shuttle vector pUEpl.Hind. A 4.3 kb cassette from the shuttle vector containing the nef gene, and BPV1 URR, was cloned further into the *Hin*dIII site of the expression vector pCGE2Hind. pCGE2Hind is derived from pCGE2 [19] by inserting the *Hin*dIII linker to the XHOI site. The final expression vector was named pCGE2-NEF (Fig. 1) and contains the nef gene under control of an RSV LTR promoter.

2.5. Purification and verification of constructs

Plasmids were transformed into competent *E. coli* cells using the One Shot Kit (Invitrogen) and grown in LB medium with 50 μg/ml kanamycin for pBN-NEF constructs and 100 μg/ml ampicillin for pCGE2-NEF, and purified using the Qiagen plasmid purification columns (Qiagen). Verification of the purified constructs was done by restriction enzyme digestion and agarose gel analyses. Dideoxy sequencing on Perkin-Elmer ABI 310 automatic sequencer was used to confirm the correct sequence of the inserted nef gene.

2.6. Cell transfection, western blotting, immunohistochemistry and replication assay

To test the Nef expression of the pBN-NEF and pCGE2-NEF constructs the plasmids were transfected to COS-1 cells using a lipid mediated transfection protocol (FUGENE® Boehringer-Mannheim) according to the manufacturer's instructions. As a negative control, COS-1 cells were transfected with pCMVβ-Gal vector containing no nef gene. Aliquots of the transfected cells were harvested at 48 and 72 hours, at 9 days and at 2 and 3 weeks. The expression of HIV-1 Nef was then assayed by Western blotting and by immunocytochemistry as described [23], using a mixture of monoclonal antibodies.

Replication of the plasmids was detected as described [19] with some modifications. Shortly, CHO cells were transfected with the plasmids in question by electroporation. Samples were taken at time points 48 and 72 hours, cells lysed and the DNA precipitated. The recovered DNA was digested with MunI and DpnI restriction endonucleases, samples run on agarose gels with MunI digested pBN-NEF as a size standard. The separated DNA was blotted on to nylon filters and hybridised with a specific probe, generated by random priming, recognising the 3′ end of the RSV LTR and Nef cDNA (Fig. 1b).

2.7. Immunisation with gene gun

Plasmid DNA was precipitated onto 1 μm gold particles using spermidine and $CaCl_2$ following the procedure in the Helios Gene Gun Instruction Manual (BioRad Laboratories). Cartridges were made to carry 0.5 mg gold and 1 μg DNA each. The amount of plasmid DNA in the cartridges was controlled spectrophotometrically. Female 6–8 week old Balb/c mice were immunised with the Helios Gene Gun on shaved and cleaned abdominal skin. For DNA delivery a helium discharge pressure of 300 psi was found optimal and used in all experiments.

Twelve mice were inoculated with pBN-CMV-NEF, four with pBN-RSV-NEF and twelve with pCGE2-NEF. Wishing to give each mouse a substantional amount of DNA and considering the limitations of the Gene Gun in this matter we decided on multiple administrations a weeks' pause was to lessen the stress on the animals. The immunisation was done six times: on days 1, 2, 3, 10, 11 and 12, and thus a total of 6 μg of plasmid DNA was administered per mouse. In order to monitor the immune response kinetics, four mice immunised with pBN-CMV-NEF or pCGE2-NEF were sacrificed at two weeks. The remaining mice from all groups were sacrificed four weeks after the last immunisation and serum samples were taken for Western blotting and ELISA and splenocytes were harvested for the CTL assay.

2.8. Detection of humoral immune response in immunised mice

To test the presence of antibodies against Nef protein in pBN-CMV-NEF, pBN-RSV-NEF and pCGE2-NEF immunised and normal mice sera, recombinant Nef (50 ng/well) coated Maxi-Sorp plates (Nunc) were incubated overnight with mice sera diluted 1:100 in phosphate buffered saline (PBS). The antigen used was provided by Prof. Volker Erfle (GSF, National Center for Environmental and Health Research, München, Germany) and the MRC AIDS Reagent Project. Plates were incubated with secondary antibody, peroxidase conjugated rabbit anti-mouse IgG in 1:500 dilution (DAKO) for 2 h at room temperature, and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, Sigma) in citrate buffer was used as the colorigenic substrate. An ELISA value (EIU) for each mouse serum was calculated using the following formula:

$$\mathrm{EIU} = \frac{(A_{\text{mouse serum}} - A_{\text{normal mouse serum}}) \times 100}{(A_{\text{max mouse serum}} - A_{\text{normal mouse serum}})}$$

The presence of anti-Nef antibodies was further confirmed by Western blotting as described earlier [24].

2.9. Cytotoxic T cell assay

In the CTL assay, the effector cells, mouse splenocytes, were activated by co-culturing them with antigen presenting cells, and the specific CTL response was detected by measuring the ^{51}Cr release from labelled target cells. For this purpose, syngeneic P815 mastocytoma (H-2^d) cells were infected with modified vaccinia virus Ankara (MVA) expressing the HIV-1 LAI nef gene and were used both as antigen presenting (stimulator) cells (APC) and as target cells. MVA is a highly attenuated replication deficient vaccinia virus, which can serve as an efficient vector for the expression of heterologous genes providing a high level of biological safety [25–28]. Infections with MVA-HIVnef were performed at a multiplicity of infection (MOI) of 5 for 16 h at +37°C [29], after which the cells were washed twice with PBS containing 10% fetal calf serum (FCS, GibcoBRL), and γ irradiated at 5000 rad. The cells were washed with culture medium before adding to the effector cells. When used as target cells in CTL assay the cells were not irradiated.

Splenic mononuclear cells from the sacrificed mice were suspended to 5×10^6 ml^{-1} in RPMI 1640 medium containing 10% FCS, 1% glutamin, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 50 μM 2-mercaptoethanol. These effector cells (5×10^6) were co-cultured with 4×10^6 of the APC described above expressing HIV-1 Nef, supplemented with 10 U/ml recombinant interleukin -2 for five days [29].

CTL activity was tested by the 4 h ^{51}Cr release assay as described [30,31]. The used effector target ratios were 50, 25 and 12,5. For spontaneous ^{51}Cr release, the target cells were plated in six wells with 100 μl of culture medium and for maximum release in six wells with 2.5% Triton-X100. After the γ counting the percentage of specific lysis of target cells was calculated as (test ^{51}Cr release − spontaneous release)/(maximum release − spontaneous release) × 100. The percentage of specific lysis ≥6% was considered to be positive as 6% was calculated to be a statistically significant difference in specific lysis compared to the control cells infected with wild type Vaccinia virus.

3. Results

3.1. Expression and replication of HIV-1 Nef

After transfection to COS-1 cells, both the pBN-NEF and the pCGE2-NEF vectors produced a strong transent Nef expression, as detected by Western blotting of lysed cells and by immunocytochemistry. pCG transfected COS-1 cells expressed HIV-Nef protein starting from 72 hours post transfection up to 2 weeks, and the proportion of Nef positive cells was 5–10%.

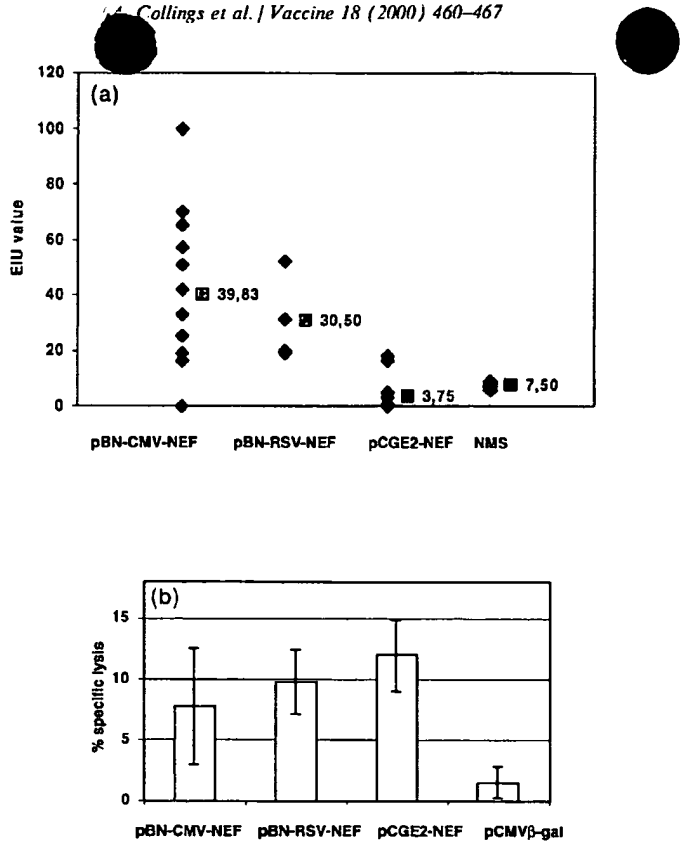

Fig. 2. Humoral and cell-mediated immune reactions of immunised mice. (a) Scattergram of the ELISA results: EIU values plotted for each group. EIU = $(A_{\text{mouse serum}} - A_{\text{normal mouse serum}}) \times 100/(A_{\text{max mouse serum}} - A_{\text{normal mouse serum}})$. Gray squares indicate means of the EIU values for each group. (b) The averages + SD of specific lysis (%) in CTL assay of mouse splenocytes four weeks after immunisation with each plasmid at the effector target ratio of 50:1.

In the cells transfected with the self-replicating pBN-vectors, Nef protein expression was seen from 48 hours up to 3 weeks post transfection. At most, 40% of the COS-1cells expressed Nef protein at day 9.

In the replication assay pBN-RSV-NEF DNA extracted at 48 and 72 hours was digested with MunI to obtain the fragment recognised by the probe, and with DpnI which cuts only dam methylated (unreplicated) DNA. The results (data not shown) indicate that the DNA extracted at 48 and 72 hours was resistant to DpnI digestion and showed a band of correct size as compared to the MunI digested control. This indicates amplification of the elements needed for Nef mRNA synthesis.

3.2. Humoral immune response

Fourteen of sixteen mice immunised with the replicating pBN-CMV-NEF or pBN-RSV-NEF constructs developed Nef specific antibodies within four weeks (Fig. 2a, Table 1). This was detectable also in the mice immunised with pBN-CMV-NEF and sacrificed at two weeks (four mice). The CMV and RSV promoter containing plasmids were equally effective in raising an antibody response. In contrast, only 2 of 12 mice immunised with the non-replicating transcription amplifying pCGE2-NEF had a weak antibody response to HIV-1 Nef in Western blotting or ELISA by four weeks and no detectable response at two weeks (Table 1).

3.3. CTL assay

At two weeks from the last immunisation with pBN-CMV-NEF, 3 of 4 mice had Nef specific CTL activity, while all mice immunised with pCGE2-NEF were negative (Table 1). After four weeks, 10 of the remaining 12 mice immunised with pBN-CMV-NEF or pBN-RSV-NEF had CTL activity, and six of the remaining eight mice immunised with pCGE2-NEF showed a cytotoxic immune response towards HIV1-Nef. (Table 1). A significant difference in the CTL responses

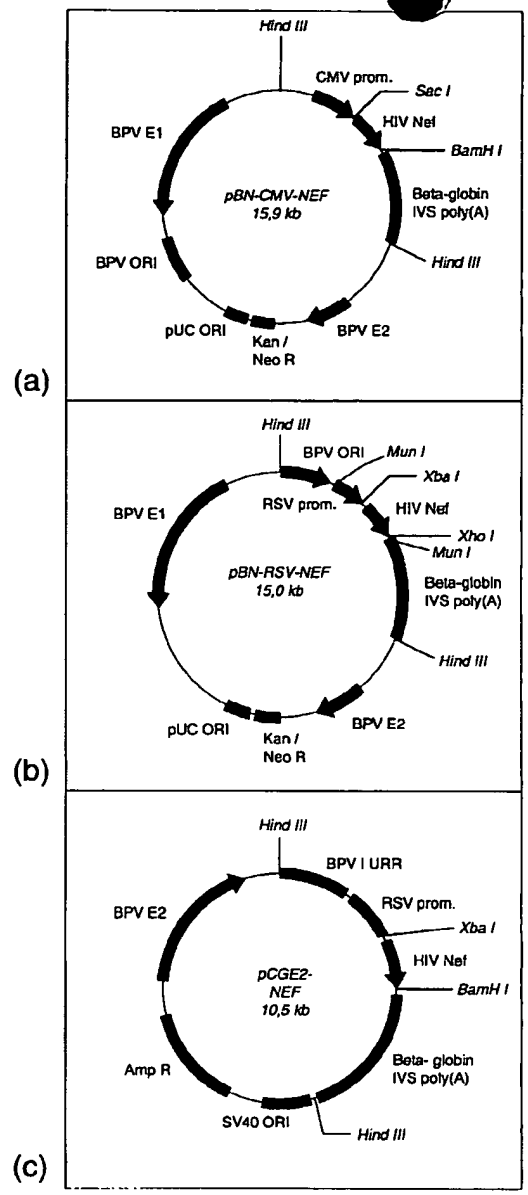

Fig. 1. Schematic cartoons of the expression vectors used in immunisation of Balb/c mice: (a) pBN-CMV-NEF, (b) pBN-RSV-NEF, (c) pCGE2-NEF.

lymphocyte (CTL) response towards HIV regulatory proteins, is associated with a favourable clinical course of HIV infection [4–7]. Methods to induce an HIV Nef-specific CTL response may thus be important in HIV vaccine development.

Live attenuated viruses are generally known to be an effective way to stimulate CTL responses but, in the case of HIV, such an approach is associated with safety problems. In contrast, naked DNA immunisation has been shown to be an effective way to induce CTL [8–10] and is thought to have no harmful effects. The vast amount of experience obtained after the first DNA immunisation reports have shown that the route of immunisation and the type of vector used for expression of the foreign gene may affect the outcome [8,11–13]. Although intradermal, intramuscular and mucosal routes have been shown to be effective in DNA immunisation an efficient and simple way to introduce genes into tissues is bombardment with DNA coated gold particles [14]. Also, it has been reported that such a gene gun based inoculation into the dermis is the most effective method of DNA immunisation [15] and induces an immune response resulting in inhibition of viral replication of lymphocytic choriomeningitis virus (LCMV) [16]. Particle mediated immunisation with a DNA vaccine to porcine Infuenza A Virus (A/Swine/Indiana/1726/88) has also been shown to protect against challenge with homologous virus [17]. Furthermore, very small amounts of plasmid DNA suffice for gene gun inoculation and induce immune responses comparable to those elicited by intramuscular or intradermal injections of DNA [18].

The most widely used expression vectors use CMV promoters, and such vectors have been applied for HIV regulatory protein immunisation as well [12,13]. However, all the vectors so far tested have had only a limited capacity of transcription and limited period of persistence. To achieve long lasting and high expression level of the immunogenic protein, we have tested the in vitro expression and immunogenicity of HIV-1 Nef using three recently developed expression vectors utilising bovine papilloma virus (BPV) regulatory elements [19,20]: two self-replicating (pBN-CMV-NEF, pBN-RSV-NEF) and one non-replicating with amplified transcription capacity (pCGE2-NEF) (Fig. 1).

These plasmids utilise elements derived from the bovine papillomavirus (BPV-1) genome: the pBN-NEF plasmids contain a BPV origin of replication (ORI) together with the E1 and E2 genes whereas pCGE2-NEF contains the E2 gene and the upstream regulatory region (URR). In one of the pBN-NEF plasmids the Nef cDNA is cloned under the CMV and in the other under the RSV promoter (pBN-CMV-NEF and pBN-RSV-NEF, respectively). The products of the E1 and E2 open reading frames are polypeptides necessary and sufficient for stable self-replication of BPV-1 ORI containing plasmids in eukaryotic cells and they are the only *trans* factors required for replication of BPV [19]. In the pCGE2-NEF plasmid, the purpose of the E2 gene is to function as a transcription activator independent of the cell context that by activating the E2 dependent enhancer in the URR [21] allows increased expression of the nef gene from the RSV promoter as has been demonstrated earlier (A. Männik and M. Ustav, unpublished). In the case of pBN-CMV-NEF and pBN-RSV-NEF, the E1 and E2 genes expressed

Table 1
Summary of the results obtained from the immunisations of mice. + and − symbols indicate a positive and a negative finding, respectively. In CTL the % specific lysis ≥6 was considered positive; the values given are at an effector target ratio of 50:1, except for mouse: pCGE2-NEF 8 where an effector target ratio of 25:1 was used. EIU values were calculated from the formula EIU = $(A_{\text{mouse serum}} - A_{\text{normal mouse serum}}) \times 100/(A_{\text{max mouse serum}} - A_{\text{normal mouse serum}})$. EIU values ≥10 were considered positive

No. of mouse[a]	Humoral response		Cellular response	
	Nef IgG	EIU	CTL	Lysis (%)
pBN-CMV-NEF 2 weeks				
1	+	51	+	6
2	+	65	+	18
3	+	57	+	13
4	+	16	−	0
pBN-CMV-NEF 4 weeks				
5	+	100	+	13
6	+	33	−	0
7	+	25	+	10
8	+	42	+	6
9	−	0	+	9
10	−	0	−	5
11	+	70	+	9
12	+	19	+	8
pBN-RSV-NEF 4 weeks				
1	+	31	+	10
2	+	52	+	12
3	+	19	−	5
4	+	20	+	11
pCGE2-NEF 2 weeks				
1	−	1	−	0
2	−	0	−	3
3	−	1	−	0
4	−	0	−	5
pCGE2-NEF 2 weeks				
5	−	5	+	10
6	−	3	+	12
7	−	1	+	16
8	+	16	+	9
9	−	0	−	0
10	+	18	+	8
11	−	0	+	10
12	−	0	−	3

[a] Also indicated the plasmid used and time of death after the last inoculation.

between the control group (pCMVβ-gal) and experimental groups is evident (Fig. 2b).

4. Discussion

Immunisation with eukaryotic expression plasmids can now be considered to be an established method to induce immune responses (reviewed in [32]). The in vivo expression of an immunogenic protein is especially efficient in inducing a cell mediated immune response including CTL and this method is therefore an applicable alternative for live attenuated vaccines.

Several parameters of DNA immunisation still await refining. Thus the used route of immunisation, the strength and length of expression of the immunising protein as well as simultaneous expression of auxiliary factors, such as lymphokines, may regulate both the quality and quantity of the obtained immune response [33].

In the present work, we tested three eukaryotic expression vectors for their capacity to induce an immune response towards HIV regulatory protein NEF. Two of the vectors (pBN-CMV-NEF and pBN-RSV-NEF) were capable of amplifying the elements needed for the initiation of replication in eukaryotic cells due to the expression of the viral replication proteins E1 and E2 functioning together with the BPV origin of replication (ORI). To drive the expression of Nef, we tested two promoters, one derived from CMV and one from RSV. As expected, no clear cut differences were observed with these two promoters either in the in vitro expression of Nef or in the immunogenic response observed in the immunised mice. The third vector, the nonreplicating pCGE2-NEF, contained an expression cartridge for the BPV1 E2 transcription activator and the E2 dependent enhancer in front of the RSV LTR, which led to the increased expression of the Nef protein.

Theoretically, the two pBN vectors were expected to produce a prolonged expression of immunogenic proteins in vitro and in vivo, and this seems to be the case. However, despite the enhancing elements in the pCG vector the in vitro expression level even at the peak of expression was not as high as that obtained with pBN. This indicates that amplification of the introduced sequences in the pBN plasmids, including the Nef coding sequence, resulting from the E1 and E2 dependent initiation of replication, has a considerable effect on the expression of the immunogen. This is also supported by the results of the replication assay in which pBN-RSV-NEF was demonstrated to replicate when transfected into CHO cells. We chose to perform the replication assay with pBN-RSV-NEF as pBN-CMV-NEF differs from this only in having a different promoter (Fig. 1) and because of its enhanced capability to amplify the elements necessary for mRNA synthesis.

Qualitatively, the form of the immune response obtained with these vectors was clearly different. With the pBN vectors, both a humoral as well as a cellular immune response were observed while the pCG vector induced only cell-mediated immunity, detectable by the CTL assay. There are several theoretical reasons for this difference. The most likely explanation is simply

the amount of foreign protein expressed with the two types of vectors. Using conventional CMV promoter driven vectors, Hinkula and co-workers have reported that cell mediated immune responses can be detected after only one inoculation with plasmids expressing HIV regulatory proteins (with as little as 1 μg plasmid DNA) while a humoral response became apparent only after booster inoculations (1 to 2 μg plasmid DNA given twice) [12]. Similar results have also been reported by Cardoso and co-workers [10]. It could, therefore, be inferred that at lower levels of protein expression the cell-mediated immune response preferentially develops while the humoral immune response is achieved only with higher levels of protein expression.

The mechanism of immune induction caused by DNA immunisation is still unknown but intriguing possibilities have been presented. Although intramuscular injection has clearly demonstrated that at least part of the DNA resides in muscle cells with subsequent protein expression, it has been demonstrated that the ultimate antigen processing and presentation to T cells takes place by professional APCs, such as dendritic cells. Several groups have shown that these APCs, located in the spleen or draining lymph nodes have taken up the expression plasmids even if DNA was injected to the periphery [34–36]. It is thus possible that even if the majority of the DNA in intramuscular or intradermal immunisations is induced to cells not capable of professional antigen presentation, such as muscle cells, part of the material finds its way to APCs and leads to a cell mediated immune response [37,38]. Regarding humoral immunity, it has earlier been shown that an antibody response is seen mostly with proteins that are secretory indicating that free native protein is required. HIV Nef is not known to be secretory, but it is possible that the high in vivo copy number of expression obtained with the self-replicating pBN vectors leads to cell destruction and release of native proteins; this could be the reason why a humoral immune response is seen with these vectors but not with the transcription amplifying pCG vector. We could not show apoptosis of transfected cells or free Nef protein in the supernatant of the cultures either in the pBN or pCG transfections. However, this is not surprising as the in vitro conditions differ from the in vivo situation where the destruction of the cells could be mediated by macrophages or other cells of the immune system.

Present experiments describe three eukaryotic expression vectors capable of inducing a cell mediated immune response of the cytotoxic type towards HIV regulatory protein Nef. Both types of vectors should be considered as a part of a genetic HIV vaccine.

Acknowledgements

The study was supported by the Medical Research Fund of Tampere University Hospital (contract number 9766); Academy of Finland; EU Science Programme (contract number BIO4-97-2109); EU Copernicus grant (grant number CIPA-CT94-0154); HHMI grant (grant number 75195-541301) and Estonian Science Foundation (grant number 2496).

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